THE DEVELOPMENT OF THE BUD IN BOTRYLLUS

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In the following account an attempt is made to describe the development of the bud in *Botryllus* in a manner directly comparable with the development of an egg, in an effort to bring out certain essential simplicities in this direct type of development. The subject itself is not new, but it is believed that the treatment is. Of the older papers those of Hjort (1896) and Pizon (1893) are outstanding. Hjort's accounts were concerned with the significance of the primary germ layers, or rather their lack of significance, in the asexual development of *Botryllus*. Pizon, on the other hand, described primarily the formation of young colonies rather than bud development as such. Also his work was marred by a completely erroneous account of the origin of the gonads, gonads being considered to arise in the oozoöid, in conformity with the opinion of Weismann, and to be transported to the developing buds of succeeding generations until sexual maturity was finally attained. This was a false conception, and the description given here is very different.

Origin and Nature of Bud

The bud of *Botryllus* first appears as a small disc-like thickening of the atrial epithelium on each side of the body, immediately anterior to the gonads. A bud appears on each side, while just posterior to them hermaphrodite gonads continue to develop. The atrial epithelium is of ectodermal origin, and apart from its involvement in the process of budding plays its part apparently only as a limiting membrane. There is, in other words, no reason to suppose that the cells of the atrial epithelium have become in any way specialized. Their formation as an epithelium is a matter of tissue organization and implies nothing in regard to cell specialization.

The disc of atrial epithelium concerned in budding is overlain externally by a similar area of epidermis. This tissue is likewise an epithelium and of ectodermal origin, but its association with test or tunicin production suggests that it has special chemical activities in addition to serving as a limiting membrane. That is, its constituent cells have probably acquired a certain degree of individual specialization.

The double disc of cells forming the initial bud is shown in Fig. 1, in optical section. In Fig. 1, *A* it is shown in relation to its subsequent developmental cycle inasmuch as three stages are shown while yet in organic continuity. In the largest and oldest of the three generations, viewed from the ventro-posterior side, the vascular connection with the circulatory system of the colony is clearly seen. The zoöid is fully active and the contained eggs have developed as far as the gastrula stage. The zoöid bears on its right side a bud about one-fifth its own length in which the organization is virtually completely expressed but is far from being functional. This bud has also formed vascular connection with the colonial system. In turn it bears a bud in the first or disc stage. Two features may be emphasized. A high degree of structural organization is attained at a relatively small size, and the size of the bud in the disc stage is minute when compared both with its size at the end of development and also with that of the developing egg. In Fig. 1, *B* the bud disc is shown, on a larger scale, in relation to the adjacent structures of the parent bud.

Polarity

The question of origin of polarity in ascidian buds has been discussed before (Berrill, 1935, 1936). In every case where organic continuity is maintained between bud and parent, and *Botryllus* is no exception, the polarity of the bud is clearly a derivative of that of the parent. Both the antero-posterior axis and the left-right axis coincide with those of the parent zoöid and must exist from the beginning.

Development of the Bud as a Whole

The simplest conception of the developing bud is that of a mass of tissue expanding during a certain period. This is shown pictorially in Fig. 2. The larger drawing in this figure represents accurately the linear growth plotted against time. The various cross-sections within the cone of growth represent certain developmental stages of special interest. At 26° C. the time units are days, and at this temperature development is completed on about the fifteenth day. If the value of the time unit is coördinated with the temperature coefficient, the cone becomes a constant expression of growth for all temperatures. The growth curve is the usual sigmoid characteristic of developing organisms in general.

At a given temperature, development to the complete functional stage has a specific duration. Equally striking is the subsequent history of the individual so formed. If the temperature is 26° C. and the developmental period fifteen days, the individual lives, feeds, and grows a

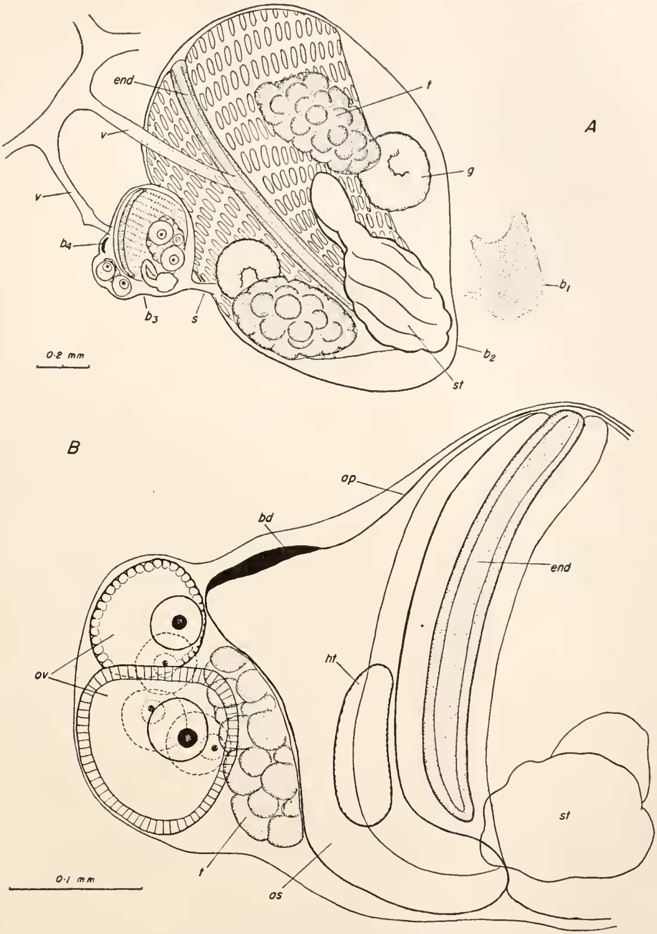

FIG. 1, *A*. Four generations representing complete cycle of zoöid. b_1, "ghost" of autolysed zoöid of preceding bud generation; b_2, ventral view of active zoöid bearing right bud only and containing developing eggs in gastrula stage. b_3, bud borne by active zoöid, with rows of definitive stigmata about to become perforate, and in turn bearing bud of next generation in its initial disc stage (b_4). Both the active zoöid (b_2) and its bud (b_3) are connected with the colonial circulatory system by their ventral ampullary vessel, *v*.

B. Part of b_3 at a higher magnification, showing general relationship of bud disc, atrial epithelium, and gonads. This stage is drawn at the same magnification as those of Fig. 4 and follows as a stage of bud development Fig. 4, *F*.

as, atrial sac; *ap*, atrial epithelium; *bd*, bud disc; *end*, endostyle; *g*, gastrula; *ht*, heart; *ov*, ovum; *s*, bud stalk; *st*, stomach; *t*, testis; *v*, ampullary connecting vessel.

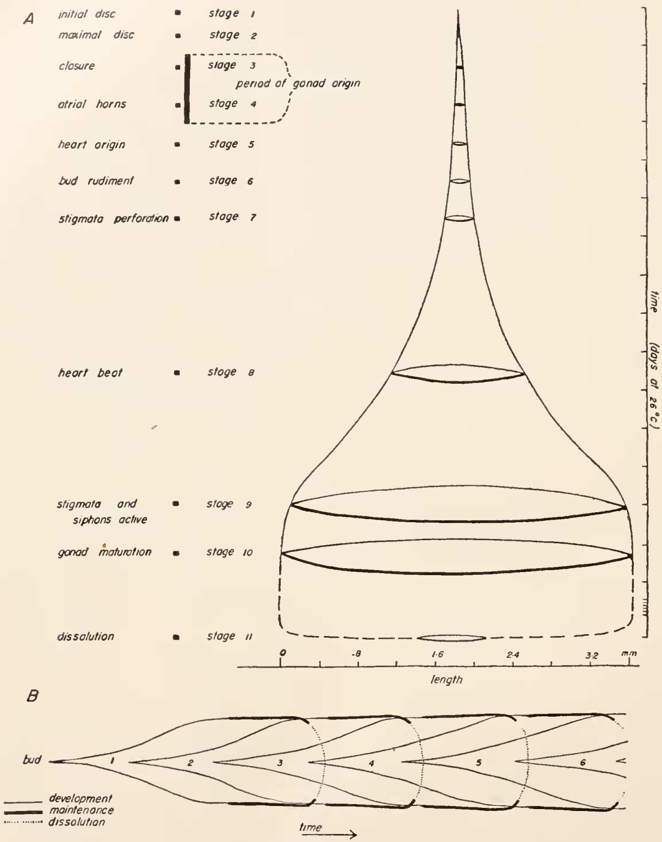

FIG. 2, *A*. Critical stages of bud development in relation to growth curve and whole life cycle of zoöid. The time scale is abbreviated for the maintenance or active period.

B. Similar growth curves for five successive generations indicating overlapping of generations and the three phases of development, maintenance and dissolution.

little, for about seven days. After this a period of dissolution and autolysis occupies a further two days. In Fig. 2, *A*, these last two phases are indicated against a condensed time scale. Their duration is related to temperature in the same way as is the first or developmental phase.

As shown in Fig. 1, *A,* the first or disc stage in bud development appears when the parent zoöid is itself but one-fifth grown and in turn still attached to its parent. In other words, there is a great extent of overlap in the life cycles of successive generations. This is shown in Fig. 2, *B.* Five complete cycles are shown in the form of overlapping growth cones related to one time scale. It can be seen that in any temporal cross-section three generations in their respective existence phases will occur. It may also be seen that the final phase of dissolution and autolysis commences immediately after the bud of the succeeding generation reaches its full size and ends at about the time the bud attains its maturity. For each generation the developmental phase is shown in light line, the mature phase in heavy line, and the dissolution phase in dotted line.

Early Development of the Bud

Throughout development the epidermis, arising in continuity with the epidermis of the parent, plays almost no part other than to form more epidermis conforming in area and shape to that of the organism arising from the atrial tissue. For the most part therefore it will be ignored.

The youngest stage so far detected consisted of eight atrial cells forming a disc of three cells, more flat than cubical, in cross-section. The whole disc was about thirty micra in diameter, and is shown in surface view in Fig. 3, *B,* and in optical section in continuity with the squamous atrial epithelium in Fig. 3, *C.* Figure 3, *A* represents an arc of the egg of *Botryllus* drawn to the same scale to show the relative size of the egg and the bud rudiment.

As the number of cells constituting the disc increases, the area of the disc increases and the constituent cells change from a sub-cubical to a columnar shape. This leads one to suspect that the columnar condition is typical but in the earliest stage the transition to the surrounding squamous atrial epithelium is so short that the columnar condition can be only partially expressed.

In Fig. 3, *D* the disc stage is shown at its maximum size. With further increase in area, or cell number, it curves into an arc, into a hemisphere, and eventually into a closed sphere (Fig. 3, *E, F, G, H*). The sphere becomes pinched off from the atrial epithelium from which it originated, and the bud remains connected with the parent primarily by an epidermal stalk. Two phases may accordingly be distinguished, an expanding disc phase, and the phase of continuing expansion during which the disc curves into a hollow sphere. A further feature of considerable significance is associated with the second of these phases. This is shown in Fig. 3, *G* and *H,* in which the gonads are already appearing. In the closed sphere stage shown in Fig. 3, *H,* the sphere proper is shown in optical section. In addition certain cells stand out clearly in surface view. These consist of three primary ova and a number of small more ventrally placed cells destined to form the testis. In the younger stage in Fig. 3, *G,* even before the sphere has closed, four cells can be seen which, from their position and shape, are undoubtedly four primary ova. These and later reproductive cells arise by extrusion or delamination from the wall of the sphere. The gonads arise therefore in a remarkably precocious manner.

In Fig. 3, *H,* the epidermis shows definite evidence of active morphogenesis, for the distal evagination is the rudiment of the epidermal stolon that unites eventually with the colonial circulatory system. The disc, hemisphere, and closed sphere stages are shown on one-half the scale of Fig. 3 in Fig. 4. In this are also shown three subsequent stages. Apart from the growth and elaboration of the gonads, which is described in detail later in this paper, these six stages represent stages in a single continuous process. The process of expansion and folding that changes a disc into a hollow sphere continues so that the sphere becomes concave along several facets or arcs. As the anterior arc continues to expand, two vertical folds appear. At the same time an evagination appears in the posterior arc. These are shown in Fig. 4, *E.* The anterior folds gradually extend posteriorly until they divide the single vesicle into a median and two lateral chambers. These three units are the central pharyngeal chamber and the pair of lateral atrial chambers (Fig. 4, *F*). At the same time the posterior evagination grows out to form the rudimentary stomach and intestine. When the primary subdivision into three chambers is complete, two small evaginations develop from the central chamber. Median and anteriorly a small bulge becomes the neural mass, while a somewhat larger evagination from the left posterior wall represents the developing heart. Therefore, apart from the segregation of lateral masses from the wall of the sphere to form the gonads, all the principal divisions of the *Botryllus* zoöid are produced, pharyngeal and atrial chambers, intestine, heart and neural complex by a simple process of progressive folding of an expanding sheet of tissue. These are all clearly shown in Fig. 4, *F.*

Later development is primarily an elaboration of detail of each of these divisions. Figure 1, *B* is of the same scale as Fig. 4 and demonstrates both the extent of growth and elaboration that occur by the time the developing atrial epithelium in turn has formed its bud disc. The

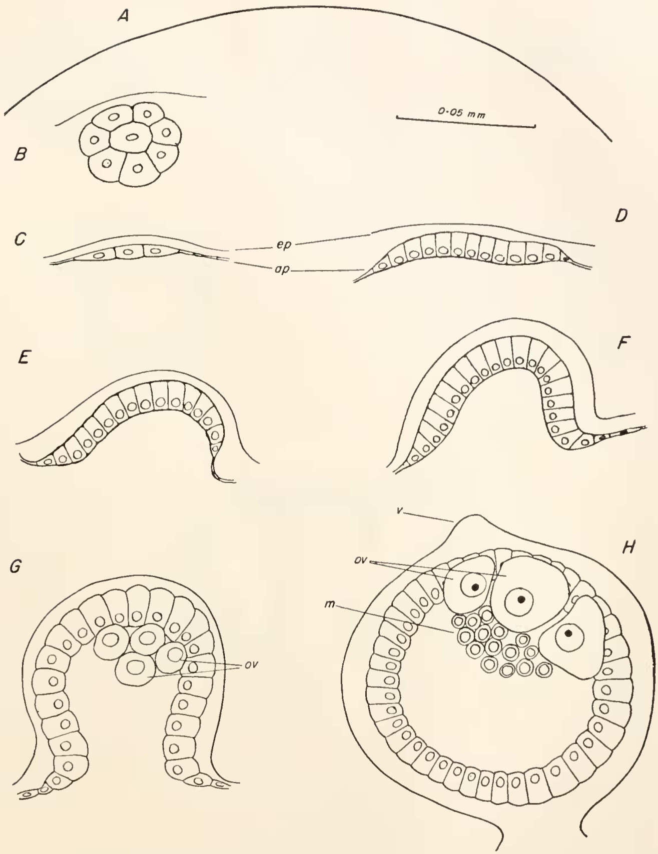

FIG. 3. Formation of bud.

A. Part of circumference of mature egg drawn to same scale for comparison of size.

B, C. Surface view and optical section of initial disc (stage 1).

D. Optical section of maximal disc (stage 2).

E, F. Arching of disc to form sphere or vesicle.

G. Bud vesicle beginning to close proximally, and showing lateral segregation of four cells destined to become mature ova, extruded into space between inner and epidermal vesicles.

H. Later stage (stage 3) with vesicle closed, epidermal ampullary vessel protruding distally, three presumptive mature ova and a number of male cells all extruded from the lateral wall of the vesicle, to lie outside it. A similar condition exists on the opposite side of the vesicle, not shown in the figure.

ep, epidermis; *ap*, atrial epithelium.

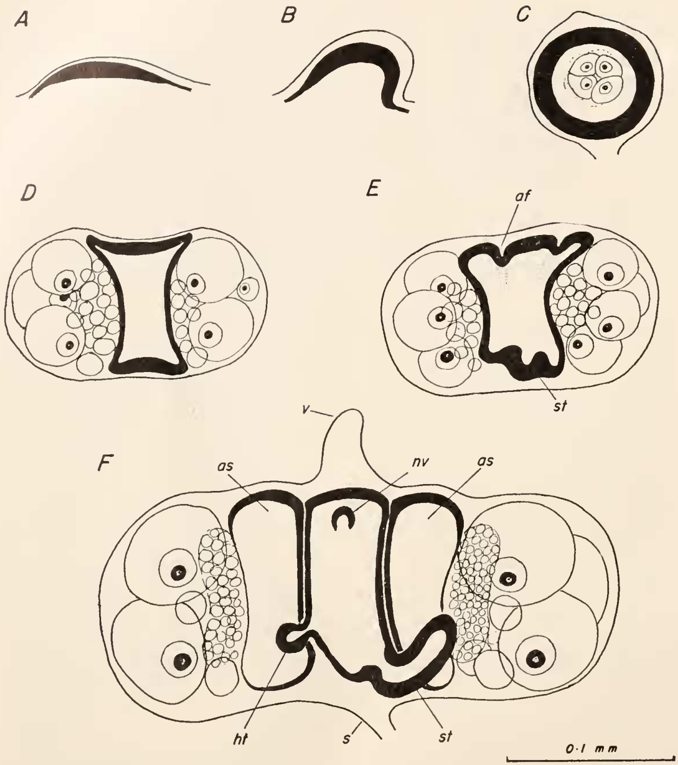

FIG. 4. Early development of bud.

A. Maximal disc (stage 2), from left side.

B. Hemisphere stage, from right side.

C. Closed vesicle (stage 3), from right side, with gonads extruded from wall.

D. Continued gonad segregation, stage viewed from ventral aspect.

E. Formation of atrial folds and intestinal outgrowth (stage 4), from ventral side.

F. (Stage 5.) Origin of heart, intestine and neural vesicle, and completion of subdivision of vesicle into central pharyngeal chamber and lateral atrial sacs, from ventral side. The succeeding stage (stage 6) is shown on the same scale as Fig. 1, *B.*

af, atrial fold; *as,* atrial sac; *ht,* heart; *nv,* neural vesicle; *s,* stalk; *st,* stomach; *v,* epidermal ampullary vessel.

bud disc appears at a precisely definable stage in the whole development and is to be regarded as an essential and definite constituent part of the organization of a specific stage.

Later Development

Development of Stigmata.—Gill slits develop as perforations of the combined pharyngo-atrial wall. This is the case both for the organism developing directly from the egg and for the developing bud. In the first case atrial sacs grow in on each side of the embryo and come into contact with the pharynx wall. Gill slits appear only within the area of contact. In the bud the equivalent double wall is formed, as already described, by the downgrowth of the pair of anterior folds that divide the primary vesicle into the central and lateral chambers, as shown in Fig. 4, *E* and *F*. The two walls are shown in Fig. 5, *A* at a stage intermediate between the preceding two. Only when this double wall expands to about ten times its linear size does stigmata formation become evident. The first indication is the appearance of an alternating thickening and thinning (spatially) of each of the two component epithelia separately, as shown in Fig. 5, *B*. The thick ridges run dorso-ventrally from the mid-dorsal line to the endostyle and each represents a row of stigmata. Between the ridges the epithelia flatten out as the interstigmal tissue. This condition is definitely associated with the stage bearing the bud disc stage of the next generation.

As the two layers of ridges or thickenings increase somewhat in depth they come into contact and fuse at a series of points along each pair of ridges. Perforation occurs at these points to form the rows of stigmata in their first definitive stage. The first perforate stage is shown in Fig. 5, *C* and *D*. Subsequent development consists of an elaboration of each of the units thus formed. No more will be added. Perforation of the fusing wall occurs at a definite and precisely definable stage of development. At this same stage other features of the developing pattern will be at a constant associated condition.

The stage of development of the bud of the next generation conforms to this relationship just as any other feature, and is near the hemisphere stage of vesicle formation (Fig. 3, *F* and *G*). In other words, the bud itself is an integral part of the whole organization pattern and the time and place of its inception are as sharply defined as that of any other unit structure in the developing organism.

At perforation each stigma in surface view consists of a rosette of about six cells (Fig. 5, *D*). Each constituent rosette of a row continues its development as a unit. With multiplication of its seemingly unspecialized cells the central aperture bordered by the cells expands and elongates, so that the size and length of each stigma increase progressively. When the cell-multiplication is almost terminated each cell develops short cilia. Further growth of each stigma to approximately

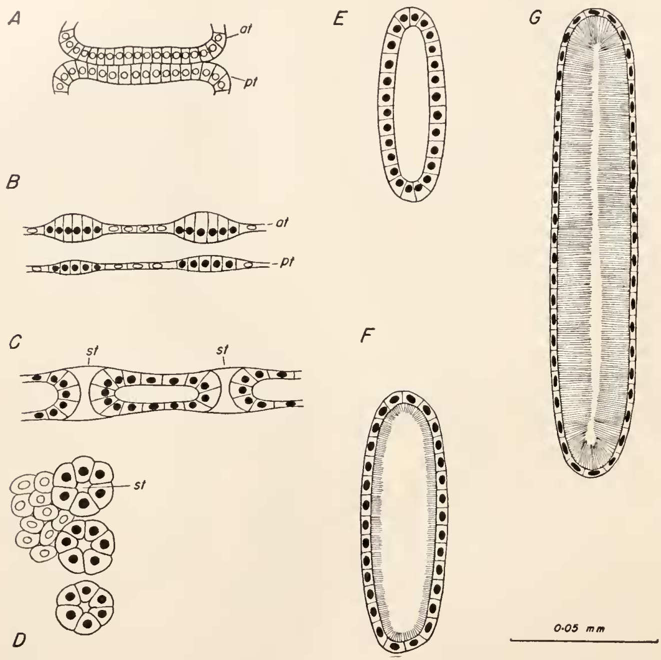

FIG. 5. Development of gill slits (stigmata).

A. Double layer formed of inner atrial wall and of pharyngeal wall.

B. Alternate thickening of atrial wall and corresponding thickenings of pharyngeal wall, each such paired thickening representing cross-section through ridge destined to become row of stigmata.

C. Equivalent section to *B,* but of perforate stage.

D. Surface view of *C,* showing three stigmata rosettes in initial perforate condition.

E, F, and *G.* Three stages in subsequent growth and differentiation of a single rosette to form a functional gill slit.

at, atrial wall; *pt,* pharyngeal wall; *st,* perforate stigmata.

double its size takes place as the result of change in cell size and shape, and at the same time the cilia grow until they almost touch those from the opposite side (Fig. 5, *F* and *G*).

Accordingly the following features are evident in the development of the gill slits: There is a primary condition in which atrial and pharyngeal epithelia are present and in virtual contact. The basic pattern is expressed as a series of ridges, each with a series of swellings in each tissue some time before stigmata formation. Perforate stigmata appear at the points of fusion between the two tissues, while subsequent growth consists first of a period of cell multiplication and then of a terminal phase of individual cell expansion and cyto-differentiation.

Development of the Gonads.—The origin of the gonads has been described already. Their subsequent development as a unit organ is, however, of some interest, as is that of a single ovum. Figure 6 shows sections through a number of stages. Figure 6, *A* represents a section of a stage immediately following that seen as a whole mount in Fig. 3, *H*. The originally thick wall of the internal vesicle is divided into the thin atrial wall and the massive developing gonad. The gonad here consists of two primary ova and a mass of loose cells representing a few rudimentary ova and many male cells. Cells are added to the collection over a considerable period from some parts of the inner retaining wall. In other words, as the lateral walls continue to grow, the splitting into inner atrial and outer gonadial components continues in marginal regions previously incapable of such splitting by virtue of insufficient cells. This is shown in Fig. 6, *B,* a section passing transversely across the anterior end of a bud at a considerably later stage, a stage intermediate between those shown in Fig. 4, *E* and *F*.

The section shown in Fig. 6, *C* illustrates several points of interest. The inner atrial epithelium is entirely distinct in kind and in space from any part of the gonad. The form of the lobular testis becomes apparent in spite of the small number of its constituent cells. And in the case of both ovary and testis there is a residual mass of cells unincorporated into those organs. In the case of the ovary, the small inner ova never grow and mature. The residual cells of the testis may or may not develop into testicular lobes, depending on the degree of belatedness of their segregation. Virtually the complete form of the testis is to be seen in the stage represented in Fig. 6, *E,* even though the testis here is less than one-quarter its final size (in linear dimension). The form is almost fully expressed, but its histo-differentiation is indiscernible. In fact the final differentiation into condensed and tailled spermatozoa occurs only after the full size of the developing bud is at last attained.

In the ovary those ova segregated from the vesicle wall in the first phase of gonad formation (in number from one to four) grow and mature. Those formed later remain close to the size at which they were segregated. The primary ova, as far as can be determined, in-

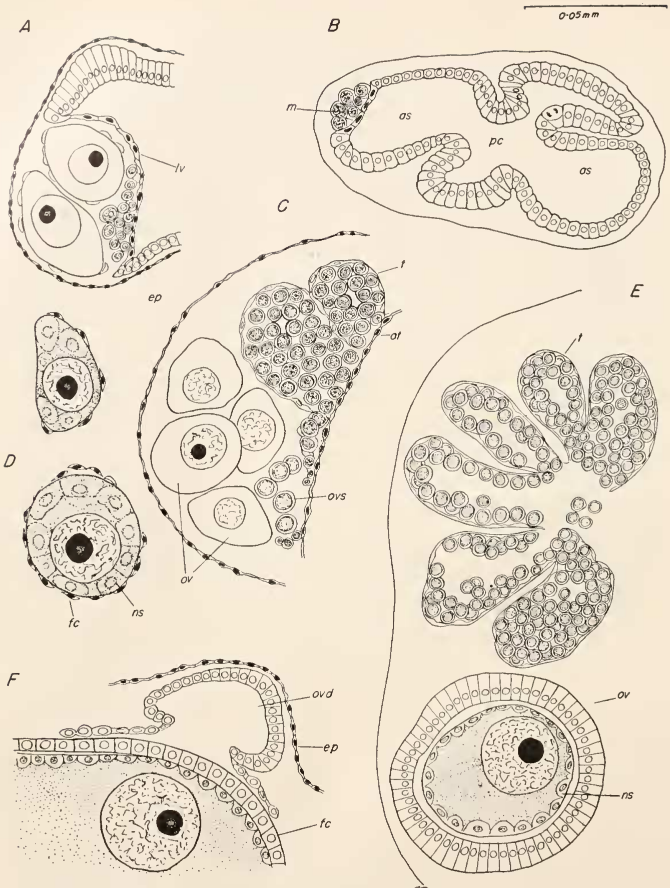

FIG. 6. Development of gonad. All drawings from actual, not merely optical, sections.

A. Left half of advanced vesicle (between stages 2 and 3) showing differentiation of lateral wall into atrial epithelium, male cells, and presumptive mature ova.

B. Frontal section of later stage (between stages 4 and 5) in anterior region, showing continued segregation on left side of male cells from the lateral wall.

C. Left side of bud at stage 5 showing four presumptive mature ova, a few undeveloping ova, and precocious lobulation of testis.

D. Two isolated ova with follicle and nurse cells.

E. Gonad of stage 6, showing lobular testis, and a single ovum with numerous follicle and nurse cells.

F. Part of ovum at its maximum size, showing nurse cells, follicle cells, and developing oviduct of same origin as follicle cells.

at, atrial epithelium; *ep*, epidermis; *fc*, follicle cell; *lv*, left wall of primary vesicle; *m*, male cells; *ns*, nurse cells; *ov*, ovum; *ovd*, oviduct; *ovs*, secondary ova; *t*, testis.

clude other cells from the first. No stage, with the possible exception of those shown in Fig. 3, has been seen in which the ova proper are without accessory cells. These cells are of two kinds, a few flattened follicle cells clinging to the surface, and an equally small number of nurse cells completely within the cytoplasm of the ovum. Following multiplication, the outer surface cells become columnar, as in Fig. 6, *D*, and eventually flatten again as they give rise to the egg chorion. The nurse cells also multiply and are clearly involved in the growth of the ovum. They are eventually extruded into the perivitelline space as the

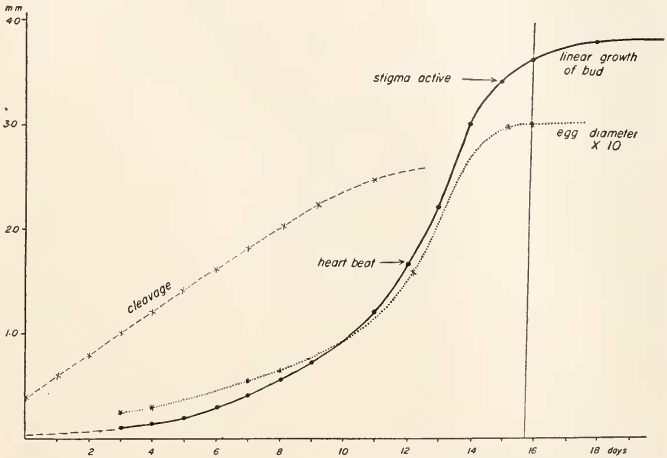

FIG. 7. Growth curves for linear growth of bud, and of single egg × 10. On the same chart is also shown a cleavage curve indicating the geometrical increase in cell number associated with growth.

inner follicle cells or "test" cells of the mature ovum (cp. Berrill, 1929).

Thus the development of the gonad as a whole is a comparatively complex process. Yet the ova and spermatozoa attain actual functional maturity at virtually the same time and almost at once after the bud as a whole has become active. Either some factors external to the gonads suddenly terminate growth and multiplication and enforce final differentiation to coincide with that of the rest of the bud, which is unlikely, or the development has from the first been approaching a condition of equilibrium permitting final cyto-differentiation. That this last is the case is shown by a comparison of the growth curves for the bud as a whole and of a single ovum. The curves for the linear growth of the bud and the ovum, when reduced to equivalent scales, are practically identical, as may be seen in Fig. 7. Both are sigmoid curves indicative of an approach to and attainment of a " steady state."

Cell Division and Differentiation.—In all tissues of which the cells finally exhibit a marked degree of structural specialization or differentiation, the structural details become visible only at or toward the close of the phase of cell multiplication. The time at which this occurs varies greatly. In the case of spermatozoa, condensation and elongation occur only after the rest of the organism has as a whole become functionally active. In the case of ova, as distinct from their associated follicle cells, growth and differentiation without division occupy almost the whole developmental period. Muscle cells of the heart and body wall stop dividing and become greatly elongate when linear growth of the whole organism is little more than half complete. Cessation of division and subsequent formation of long cilia in stigma cells occurs very late, but is complete before elongation of spermatozoa commences. In the case of ova and muscle cells, at least, there is very considerable growth after cell division has come to an end. Growth of the organism as a whole accordingly conforms to a typical curve in spite of the fact that the growth is in part due to cell multiplication and in part to cell growth without division. The growth curve for a single ovum is similar to that of the whole organism. It seems clear that the developing bud grows at a rate characteristic of an approach to a " steady state," and that the growth of the parts, whether based on cell division or not, is not a group of independent processes coöperating to form the whole, but must be governed by the whole. Cell division as such becomes, in this view, a condition and tool of the whole developmental process rather than in any sense a basic cause. Otherwise the coördination of the varying times at which different cell types cease division and differentiate becomes virtually unaccountable. In a comparison of the growth of a non-dividing ovum with the growth of a group of cells by multiplication, it appears that the rate of volume-increase is quite independent of division processes.

Summary and Conclusions

The bud arises as a disc-like thickening of the anterior atrial wall, consisting of a small number of columnar cells transformed from the atrial epithelium, overlain by an equivalent area of unmodified epidermis. The polarity of the disc and subsequent organism is an extension of that of the parental tissue, with regard both to the antero-posterior and lateral axes. Development itself is fundamentally extremely simple and direct. After the completion of development there is a phase of functional activity and a phase of autolysis and dissolution. For any given time-temperature scale the duration of these last two phases is as specific and determined as that of the developmental phase. Of the two tissues constituting the bud disc the epidermis forms only more epidermis, though acquiring the form of a whole organism including the ventral stolonic outgrowth. The atrial component of the disc forms everything else. As the disc expands, by means of cell multiplication, it transforms progressively into a hemisphere and eventually into a hollow sphere attached by a narrow stalk to the parental tissue. Two folds develop anteriorly and divide the vesicle into two lateral and one median chamber. The lateral divisions represent the atrial chambers, the median the pharyngeal sac and from it three evaginations are formed representing the heart, neural mass, and intestine respectively. Later development is primarily an elaboration of these unit-regions. As an example, the formation, growth and differentiation of gill slits in the pharyngeal wall is described in detail. The essential pattern of the stigmata is apparent even before they become perforate. Each stage in their development is precisely correlated with specific stages in the development of the whole organism.

The bud anlagen of the succeeding generation appear as discs in the anterior wall of the left and right atrial chambers at a specific stage in the development. This stage is that in which rows of stigmata, while not yet perforate, are represented by ridges or folds of the pharyngeal wall. At the time of perforation, the buds are approximately at the closed vesicle stage. The buds, in fact, are to be regarded as essential constituents of the organization pattern, appearing and developing in time and place in a manner strictly analogous to that of any other unit structure.

The gonads segregate as a mass from the lateral walls of the bud at an extremely precocious period, even while the primary vesicle is in process of formation. Once segregated, they in turn develop as a seemingly independent unit structure. The testes show the final lobular form virtually as soon as sufficient cells are present for its expression. Ova, apart from the associated internal and external follicle cells, grow and differentiate without dividing. They mature finally at the same time as the spermatozoa which cease dividing and differentiate later than any other tissue of the bud.

The development of each tissue is fundamentally the same. A period of cell multiplication is followed by a phase of final differentiation. This last phase may or may not include a period of cell enlargement, depending on the cell type to be formed. In the case of ova the multiplication phase is barely present at all and the second phase occupies most of the developmental period, involving enormous growth. In spermatozoa the case is reversed and the final phase is extremely brief and actually involves reduction in cell size. Muscle tissue lies between these two extremes, while most other tissues approach more the condition of spermatozoa.

The whole development of the bud and that of its component parts is therefore as direct a process as can be conceived, without there being any indication of the divergence to form tadpole larvae associated with egg development. Cell multiplication continues to a greatly varying extent in different parts and tissues, while the linear growth of the whole or of a non-dividing ovum follows a regular sigmoid curve typical of an approach to and attainment of a " steady state." In fact, the development of the bud is essentially such a unitary process that " wholeness " can be said to be the most outstanding feature of the organism not only in its final functional phases but of every moment of its existence, and especially of the beginning. It is virtually as though organization is present from the first, though the extent of its visible expression is closely correlated with and limited by quantity of available material at every moment of development.

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